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STUDIES OF BACTERIOLOGICAL AGAR

I. Physical and Chemical Properties
By Leonard S. Stoloff and Charles F. Lee 1/

II. Bacteriological Studies
By Leslie A. Sandholzer, Margaret E. Dean,
William Arcisz and Martha C. Bradley 1/

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INTRODUCTION

During World War II, all agar was reserved for scientific purposes, principally bacteriological. This was necessitated by the shortage caused when we could no longer receive imports from Japan which had supplied 92 percent of our agar. To help alleviate this condition, the U. S. Fish and Wildlife Service undertook an investigation of the properties of agar and agar substitutes relating to their use for bacteriological purposes.

Agar, U.S.P. is defined in the Pharmacopoeia of the United States XII (1942) as "the dried mucilaginous substance extracted from Gelidium corneum and other species of Gelidium and closely related algae." The War Production Board (1942) expanded the definition of agar to include "any mucilaginous substance, whether dried or in other form, extracted from Gelidium corneum, Gelidium cartilagineum, Gelidium amansii, Gracilaria confervoides, Gracilaria lichenoides, Eucheuma spinosum, Eucheuma isiforme, Eucheuma denticulatum, Gigartina spinosa, Gigartina mamillosa and from other species of the genera named above and closely related algae of the class Rhodophyceae."

In commerce, and particularly in bacteriology, the term "agar" has achieved a more restricted meaning with regard to the physical properties expected. The diversity in physical properties of the gums meeting the source definitions was made apparent when wartime trade restrictions turned our attention to seaweeds not normally used in the preparation of agar.

Table I (Stoloff, 1943a) indicates some of the seaweeds which have been reported as sources of agar or agar-like gums.

Variations encountered in some of the physical properties of a number of seaweed extractives, as indicated in Table 2, and the nature of some of the agar substitutes recommended in the literature (McReady, et al. 1943; Humm, 1942; Tschudy, et al. 1943; Baier and Manchester, 1943; Zimmerman, 1939, and Funck, 1937) make it desirable to define more closely what is meant by agar for bacteriological use.

A search of the literature revealed no precise definition of the bacteriological requirements of agar. The Difco Laboratories (1943) had established certain criteria of purity for their product, and Noble (1928) and Noble and Tonney (1935) had specified a highly purified agar for certain culture media. The Pharmacopoeia of the United States XII (1942) also contained a definition of purity for agar intended for pharmacological use. This definition is inadequate for bacteriological agar. The United States Army specification for bacteriological agar (No. 4-1041-1935) calls for agar with "---little or no color, odor or taste. ---clean and free of foreign matter. Insoluble matter, not to exceed 0.1 percent by weight." These qualifications would also be inadequate if it were not that the product "Shall be ---- free from any imperfections which might affect either the appearance or purpose for which it is intended." The need for so broad a statement emphasizes the desirability of limitations for physical properties and chemical impurities of bacteriological agar.

As long as the extracts used for this purpose were obtained primarily from Gelidium these qualifications were satisfactory, but they did not permit an evaluation of the suitability of other seaweed gums for bacteriological use.

TABLE 1. Seaweeds reported as sources of agar

Seaweed	Reported occurrence	Remarks
Ahnfeltia plicata	Russia (Maritime Coast and White Sea)	Maritime grade best.
Camphylaephora hynaeoides	Japan, China	
Endocladia muricata	California	Reported originally by Field (1921).
Eucheuma spinosum	Japan, China	Reported used to a considerable extent in these countries.
isiforme		
denticulatum		
Gelidium amansii	Japan, China, California	These seaweeds are main source of agar in Japan, China, and California.
cartilagineum	Japan, China, California South Africa	
corneum	Japan, China, California	
australe	California	Gel similar to Irish moss.
pristoides	South Africa	Gum difficult to dissolve, low gel strength.
Gigartina asperifolia	California	These seaweeds require special treatment to obtain gum.
canaliculata		
serrata		
Gloeopeltis	California	
Gracilaria confervoides	Japan, China, Australia, South Africa, California, North Carolina, British Columbia	This seaweed is main source of agar in Australia, and South Africa.
lichenoides	Japan, China, Australia	
Phyllophora nervosa	Russia (Black Sea)	
rubens		
Pterocladia lucida	Australia	This genus is closely related to *Gelidium*.
capillacea	Brazil	
sp.	California	
Suhria vittata	South Africa	Gel similar to Irish moss.

TABLE 2. Data on properties of extracts of a number of Rhodophyceae

Product	Concentration	Breaking Strain	Gelation 1/ Temperature	Total ash, dry weight basis	Viscosity at 50°C.
	Percent	(gm./cm.) 2/	(°C.)	Percent	Centipoise
Chondrus crispus extract (commercial, improved)	3 5	30 90	31	30.7	10 --
Digenea simplex extract (Florida)	3	0	--	40.0	10
Hypnea musciformis extract (Florida)	3	10	--	26.7	55
Hypnea musciformis extract (North Carolina)	1½	0	44	43.4	very viscous
Gelidium nudifrons extract (California)	3	20	--	25.9	101
Gracilaria confervoides extract (North Carolina)	1 1½	90 120	59 and 43	3.4	--
Gracilaria confervoides extract (Australia)	1½	50	46	4.8	--
Gracilaria confervoides extract (California)	1½	50	47	--	--
Gracilaria cornea extract (Florida)	2 3	3 5	46	6.7	108
Gracilaria floridana extract (Florida)	2 3	30 60	-- 48	-- --	-- very viscous
Gracilaria multipartita extract (North Carolina)	1½	50	53	26.1	very viscous
Gracilaria sp. extract (Brazil)	1½	20	43	13.5	8

1/ Approximate temperature at which appreciable gelation occurs.
2/ Grams per centimeter of plunger circumference.

In order to plan an effective research project, various members of the laboratory staff conferred with members of committees of the Society of American Bacteriologists, the American Public Health Association, the industry, and interested federal and state agencies. From the ideas and suggestions received, a coordinated project was outlined. This report deals with the work done on the several aspects of this project. The first section is limited to a study of the physical and chemical properties of agar, although the methods described may be used in studying other seaweed extractives. The second section deals with bacteriological studies. The work was done in 1943 and 1944.

I. PHYSICAL AND CHEMICAL PROPERTIES

Introduction

Before it can be determined which physical and chemical properties are applicable for defining agar, and what impurities require restrictive tolerances, inquiry should be made into the nature of the material. As broadly defined, agar is a gum common to many members of the Rhodophyceae. It is extracted from the plant with boiling water followed by filtration, sometimes through an absorbent material to remove impurities such as proteins and pigments. The most common procedure for concentrating the gum is by freezing and subsequent thawing, a portion of the soluble impurities being removed in the water which drains off in the process. The gum is left as a wet, spongy mass which may be washed and bleached before drying or can be dried directly. The dried agar may be in flakes, shreds or sheets depending on the manner in which the preceding operations have been conducted, and can be ground to any particle size for convenience in handling and packaging.

The final moisture content of the dried agar is dependent on the relative humidity and temperature of the atmosphere to which it is exposed and may vary from approximately 4 to 43 percent by weight (Stoloff, 1943b and Browne, 1922). Under the normal climatic conditions of the temperate zones, the moisture content of agar will be from 12 to 20 percent by weight. Agar also contains small amounts of water soluble impurities such as salts, proteins, and carbohydrates which were present in the original extract. Other physiologically important organic compounds may also be present (Day, 1942; Itano, et al. 1934; Robbins, 1939 and Robbins, et al. 1942). The amounts of soluble and insoluble impurities depend on the methods and the care used in processing.

The principal constituent of agar is a sulfuric acid ester of a galactan, according to Fairbrother and Maston (1923), Neuberg and Ohle (1921), Waele (1929), and Hoffman and Gortner (1925). Its general structure has been given by Jones and Peat (1942). Because of the ester structure, some of the determined ash is part of the molecule. Purification of commercial agar has shown this bound ash to be between two and three percent of the dry agar by Fairbrother and Maston (1923), and Stoloff (1943b). Small quantities of pentosans are also present according to Fellers (1916). Hoffman and Gortner (1925) found the average molecular weight to be about 3000, but the length and composition of the molecule are probably variable as evidenced by a gradient in solubility and gelation temperature of homogeneous samples and the ability to wash out the more easily soluble fractions, leaving the more slowly soluble fractions having a lower ash content and greater gel strength. The physical properties of the molecule depend on the salts of the ester, since the free acid, which has a pH of 2.5 in an 0.8 percent solution does not gel. Neutralization with any base, even an organic one, will restore this property according to Waele (1929) and Hoffman and Gortner (1925). The basic constituents normally present in the ash have been determined by various workers, and these data are summarized in Table 3. Waele (1929) reports that it is possible to replace the sulfuric acid with other inorganic acids without loss of the gelling property. Hydrolysis of the ester, which is easily accomplished by heat in acid solution, will destroy the characteristic physical properties of agar gels,

as will also hydrolysis between the two carbohydrate fractions of the agar molecule. The presence of an easily hydrolyzed linkage of this type has been reported by Takahashi and Shirahama (1934), and it is stated to be more easily hydrolyzed than the sulfuric ester, which is a component of one of the fractions.

TABLE 3. Data on the mineral constituents of agar ash

Element	Percent in ash - calculated from the data of:			
	Fellers (1916)	Kitzevetter (1937)	Forbes, et al (1913)	Whittaker (1911)
Calcium	11.2	27.4	15.6	16.4
Magnesium	5.8	6.5	11.4	8.1
Sodium	3.2	3.5	2.7)	4.5
Potassium	0.9	---	2.6)	
Iron	6.8	1.1	---	4.3

The characteristic physical properties of agar are its ability to absorb large amounts of water, dissolve in water when heated, and form thermally reversible gels at low concentrations. The features desired in agar sols for bacteriological work are low viscosity, the ability to remain liquid at temperatures that will not kill the inoculum, and the ability to set to a firm gel at room temperature. Desirable properties of the gels should be clarity, strength, lack of appreciable syneresis, and high melting temperature.

Through the courtesy and cooperation of the War Production Board, Defense Supplies Corporation and the Difco Laboratories, 73 representative samples of agar were supplied from the national stockpile. These have been analyzed to determine the range of concentration of characteristic impurities of agar, and the properties of agar sols and gels. Three agar samples, prepared especially for bacteriological use were included for comparison.

Methods of Analysis

Since the moisture content of the agar samples was dependent on the relative humidity at the time they were packed (Stoloff, 1943b) an analysis for moisture in this report serves only to provide a dry weight basis for the comparison of all subsequent determinations. The moisture content of individual samples of agar would be of importance, inasmuch as prices on bulk purchases should be based on dry weight in view of the large variation possible. The methods used for determination of moisture and other properties studied will be given in sufficient detail to enable duplication of results.

Moisture was determined by drying a 2 to 3 gm. sample in an air oven at a temperature of 160°C. for 1 hour. Dishes were covered immediately, and weighed rapidly as soon as cool, as the agar has a strong tendency to re-absorb water (Stoloff, 1943b). The temperature of 160°C. did not result in detectable decomposition of agar although other seaweed gums are less stable, and for these lower drying temperatures are necessary.

Protein content of agar is an index of purity and from a bacteriological standpoint must be considered as a source of nitrogen from a supposedly inert constituent of the culture media. Protein (N x 6.25) was determined by the usual Kjeldahl technique, using a five gm. sample and copper sulfate as a catalyst.

Water insoluble debris, a measure of the gross impurities present in agar, is an indication of carelessness in processing, particularly in filtration. For its determination a sufficiently large sample to be representative, 500 gm. of a 1.5 percent (dry weight) sol was prepared. The agar was dissolved by heating in an autoclave for 20 min. at 15 pounds steam pressure. A 100 gm. portion of the well-mixed solution (the debris tends to settle out) was diluted to approximately 300 ml. with hot water and reheated to near boiling to facilitate filtration. A Gooch crucible with a thin asbestos mat was preweighed and heated before use to prevent momentary formation of a gel which would not redissolve and which would thus render filtration extremely slow. For the same reason the Gooch crucible was not allowed to become dry at any time, the cold air, which would be pulled through, forming a gel in this case. The beaker and crucible were flushed several times with hot water and the increase in weight of the redried crucible was due to insoluble debris.

Total ash content is influenced by the manner in which the product is prepared, and by the average size of the agar molecule. Only a few of the seaweeds listed in the definitions yield gum extracts characterized by large molecule size and relatively low ash, so that the ash content in conjunction with other indices of purity can show the types of seaweeds used. These gums generally have the more desirable properties from a bacteriological standpoint. Total ash content was determined by igniting samples in platinum dishes in a muffle furnace at 550°C. until the disappearance of all black particles.

Acid insoluble ash, which is primarily an indication of the efficiency of filtration of the seaweed extract, was determined by leaching the total ash with one-normal hydrochloric acid, catching the insoluble residue on an ashless filter paper and igniting in the same platinum dish and in the same manner as for total ash. Dish and filter paper were thoroughly washed free of chlorides with hot distilled water. The filtrate was tested with silver nitrate.

The normal ash constituents, calcium, magnesium, sodium and potassium, would have little if any physiological significance. However, iron and copper are accumulated to a variable extent from equipment used in the processing of agar. There is a possibility that large amounts of these elements are detrimental in certain special bacteriological tests. Consequently, quantitative determinations of these elements were made on the acid soluble ash.

Iron.--The filtrate from the acid insoluble ash was made up to 100 ml. and iron was determined colorimetrically with potassium thiocyanate using a 20 ml. aliquot. One ml. of a saturated solution of potassium persulfate was added to insure oxidation of the iron. The intensity of color developed was measured with an Aminco photoelectric colorimeter using a 420 millimicron filter and was compared with a standard curve to determine the iron concentration.

Copper was determined with a 20 ml. aliquot of the same solution used for the iron determination. A colorimetric procedure using sodium diethyldithiocarbamate was employed (Coulson, 1937). The color developed was extracted with carbon tetrachloride, the extract dried with a small amount of anhydrous sodium sulfate, filtered, and the color measured in an Aminco photoelectric colorimeter using the 420 millimicron filter. Considerable care is necessary in the adjustment of the pH and at least two extractions are required in order to obtain quantitative extraction of the yellow color. The amount of copper was determined by comparison with a standard curve prepared by extraction of known amounts of copper in exactly the same manner.

Color is also an indication of carelessness or mishandling in processing. Some color is extracted from the seaweed but excessive color is usually due to scorching or prolonged heating and to foreign materials. Color was determined with a second portion of the 1.5 percent sol prepared for determination of insoluble debris. After the hot sol had been filtered through absorbent cotton, three drops of concentrated hydrochloric acid were added and it was reheated to hydrolyze the agar. The protein coagulated and carried down any remaining suspended matter with it. The color of the clear supernatant liquid resulting is due only to soluble pigments and was compared to distilled water in an Aminco photoelectric colorimeter using the 420 millimicron filter. All pigmentation is of the same type, shades of yellow to brown, and this filter was found to give the maximum absorption. In the absence of any absolute units, the color was expressed as density which equals log (Io/I).

Transparency.--Included in the insoluble debris may be semi-colloidal material not readily removed by the usual practice of filtering culture media through absorbent cotton. This type of material affects the transparency of the gel. The structure of the agar gel also serves to scatter light. The extent of this scattering or rather the transparency of the gel, was estimated by a modification of the Army procedure for determining opacity (Spec. No. 4-1044, 1935). A 1.5 percent (dry weight) sol of the agar was filtered through absorbent cotton and poured to depths of 2.0 and 3.0 cm. in 250 ml. beakers. The gels were allowed to set and age overnight at room temperature, after which the smallest size of "newsgothic" print (American Type Founders) which could be read through the gel was determined. The two depths were used so as to cover the observed range of transparencies with the sample page of print, running from 6 to 72 points. From those samples which had transparency values measurable at both depths from the page of print, it was determined that through two cm. of gel, print one-sixth the size of that discernible through three cm. of gel could be read. This ratio was used to calculate the sizes of print less than six point that might have been read through two cm. of gel.

Certain of the physical properties of agar are generally characteristic of its source. As has been mentioned previously, it is possible to increase the gel strength by washing out the more easily soluble fractions, but in the manufacture of agar this occurs unintentionally, if at all. In the preparation of agar there is, however, some degree of hydrolysis, and because of this and differences in raw material and methods used, differences in gel strength are to be expected.

Gel strength, or more accurately, breaking load was determined with gels of 1.5 percent (dry weight) concentration. Many of the possible variables affecting gel strength were studied and the standard conditions necessary for reproducible results were established by Stoloff (1948a). The 1.5 percent sol was prepared by heating the agar and water in an autoclave at 15 lbs. steam pressure for 20 min. It was then adjusted to the proper weight with distilled water, mixed thoroughly, and poured to the maximum depth in crystallizing dishes 7.0 cm. in diameter by 5.0 cm. deep. Samples in this series were poured in triplicate and unusual values were checked on a second set of three. After the gels had set, the dishes were covered to keep off dust and prevent excessive evaporation, and aged for at least six hours at room temperature. They were brought to some standard temperature, 30°C. in this series, in a water bath or incubator, and the breaking load was determined. The dish containing the gel was placed on the pan of a spring scales of one kg. capacity with an adjustable dial which was now set to read zero. A load was applied to a plunger, either mechanically or by hand, until the plunger ruptured the gel surface, and the maximum pointer deviation was read. Gel strength values were calculated in terms of grams per centimeter of plunger circumference.

The plunger used may be any round rod of about $\frac{1}{2}$ in. diameter faced off squarely at one end but with the edge slightly rounded to prevent cutting of the gel. A means by which the load may be applied to the plunger at a regulated rate of approximately 50 gm. per second is desirable but not necessary.

Viscosity of agar sols is affected by concentration and by the presence of electrolytes, according to Clayton (1932). For practical purposes it is possible to consider the ash impurities as part of the agar, and to determine the viscosity of the sols at the 1.5 percent (dry weight) concentration and temperature of 45°C., at which plates are usually poured in bacteriological practice. Viscosity of the samples was measured with a MacMichael viscosimeter using a wire calibrated with standard sucrose solutions (Bingham and Jackson, 1917). Actually the viscosity of most agar sols varies but little with decrease in temperature down to the transition point from sol to gel, when a marked increase in viscosity occurs according to Clayton (1932) and Stoloff (1943b). This is in marked contrast to the behavior of the solutions of some of the other seaweed extracts shown in Table 2.

Temperature of gelation.--By observing the viscosity and watching for the appearance of flocculi as an agar sol is slowly cooled, the temperature at which gelation first begins can be noted. This temperature is independent of

the agar concentration, and is from 38° to 42°C. for most agars (Gelidium). However, at this temperature gelation is neither instantaneous nor complete. There is a range below the initial transition temperature in which more and more of the agar precipitates out to form the gel. The determination of the initial transition temperature, and the onset of real gelation is long and tedious, and it is sufficient for the bacteriologist to know whether or not gelation occurs at or near 40°C. This was easily determined by immersing tubes of the agar sol in baths at the desired temperatures and noting the presence or absence of gelation after a given time. Since it is desirable that agar sols remain liquid at 45°C. and yet set to a firm gel at the highest temperatures encountered during summer weather, baths equipped with thermostats set at 35°, 40°, and 45°C. were used. The conditions of the tube contents were noted after five hours in the 45° bath and after one hour in the baths of 35° and 40°C. When gelation occurred, it was noted whether the gel was firm, weak, or very weak.

Syneresis.--The extent of syneresis of gels is of great interest to the bacteriologist because an excessive amount of surface water makes colonies spread and causes gels to slip in their containers. Most true agars are satisfactory in this respect but gels from other seaweed extracts frequently exhibit excessive syneresis. The method developed by Stoloff (1948b) was used to measure syneresis. Agar sols of 1.5 percent (dry weight) concentration, made up in the same manner as for gel strength measurements, were allowed to cool to 45°C. before pouring 100 ml. aliquots into 250 ml. Erlenmeyer flasks. With some seaweed extracts it is necessary to pour aliquots at higher temperatures but the sol should be as near the gel point as it is possible to handle, so as to minimize the condensate on the flask walls above the gel surface. The flasks with gel were cooled in a 20°C. incubator for one hour and then stoppered and stored for at least 24 hours in a 37°C. incubator before syneresis was measured.

The water was separated from the gel surfaces by washing the gel in the flask with 5 or 6 portions of carbon tetrachloride (saturated with water) of 10 to 15 ml. each. Each portion was swirled vigorously over the gel surfaces before pouring it into the measuring container. A pear-shaped centrifuge tube of 125 ml. capacity, with tip graduated to 3 ml. in tenths of a ml. (A.S.T.M. oil tube) was found convenient for this purpose. The centrifuge tube was completely filled with carbon tetrachloride, stoppered and then centrifuged in an inverted position until the water was completely separated. The amount of water, now in the tip of the tube, was read directly from the graduations. Syneresis is reported as the number of milliliters of water separated under these conditions. Values reported are averages of three gels but unusual values were checked with a second set of three gels.

Results

One feature of almost any data on the physical properties and chemical constitution of seaweed gums is the very wide variation encountered for extracts from comparatively closely-related seaweeds. This is indicated in Table 2, in which are presented data on 12 seaweed extracts, of which 7 are from 4 different species of Gracilaria and 4 from other East Coast genera,

with 1 sample from a West Coast Gelidium, species nudifrons. Breaking loads encountered ranged from 0 to 120 gm. per cm., total ash from 3.4 to 43.4 percent, and viscosity at 50°C. from 8 to measured values of 108 centipoises, with several samples too viscous to measure. In Table 3 are data on ash constituents calculated from reports of four different investigators. Although these data pertain to agar only, the percentage of calcium in the ash ranges from 11.2 to 27.4, of magnesium, from 5.8 to 11.4, and of iron from 1.1 to 6.8 percent. Only sodium and potassium appear to be relatively constant, totaling 4 to 5 percent of the ash. It is not surprising then, with 73 samples tested and reported in this paper, that a wide range of values was observed for most of the properties studied.

Trade designations indicated that the samples submitted were from two sources, Kobe and Yokahama, and that from each source there were two grades, namely 1 and 3. There were 44 samples of Kobe grade 1 from 13 lots and 8 samples from 3 lots of Kobe grade 3. Yokahama grade 1 was represented by 18 samples from 5 lots and Yokahama grade 3 by 3 samples all from 1 lot.

Samples were presumably selected from bales in a large stockpile on a strictly random basis, although no definite information in this regard is available.

The total data without regard to source or grade have been summarized in the form of histograms (Figures 1 to 12) from which the peak frequency, range and distribution are apparent at a glance. There are, however, definite indications of an influence of grade or source upon certain of the variables studied, and these will be subjected to a closer examination and discussion.

Protein content (Figure 1) shows a rather broad frequency peak in the intervals representing 1.4 to 1.7 gm. per 100 gm. of dry weight of agar (hereinafter referred to simply as percent) with 86 percent of the samples in the range from 1.3 to 1.8. The protein content of scattered samples ranged up to a maximum of 4.16 percent, but the samples analyzing more than 1.9 percent protein were exclusively from the grade 3 lots.

Water-insoluble debris is related to grade, although Figure 2 shows no definite peak frequency in the range from 0.3 to 1.1 percent. Samples analyzing 0.3, 0.6, and 0.9 percent insoluble debris occur with equal frequency. The grade 3 samples contained considerably more debris with this extraneous material in one dirty sample analyzing 7.8 percent of the dry agar.

Total ash content (Figure 3) has a high frequency distribution in the range 3.8 to 4.0 percent with 58 percent of all samples analyzing under 4.2 percent. The ash contents of the remaining samples vary rather uniformly up to a maximum of 7.2 percent. There is a low peak of frequency at 4.8 to 5.0 percent. Examination of the data in Table 4 shows that Kobe grade 1 samples were of uniformly low ash content, a maximum of 4.44 percent, while Yokahama grade 1 samples averaged much higher, only 2 samples out of 18 containing less than 4.55 percent total ash. Kobe grade 3 agars also analyzed high in ash but this was to be expected as a result of the impurities already evidenced by the high protein and debris content of these agars.

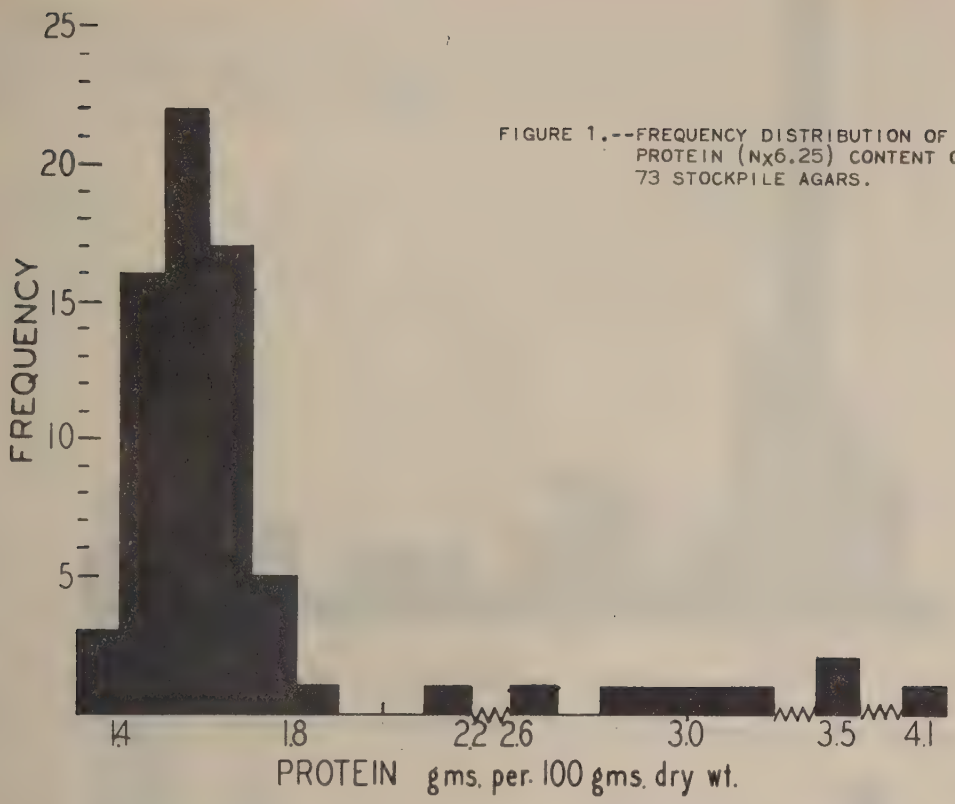

FIGURE 1.--FREQUENCY DISTRIBUTION OF PROTEIN (Nx6.25) CONTENT OF 73 STOCKPILE AGARS.

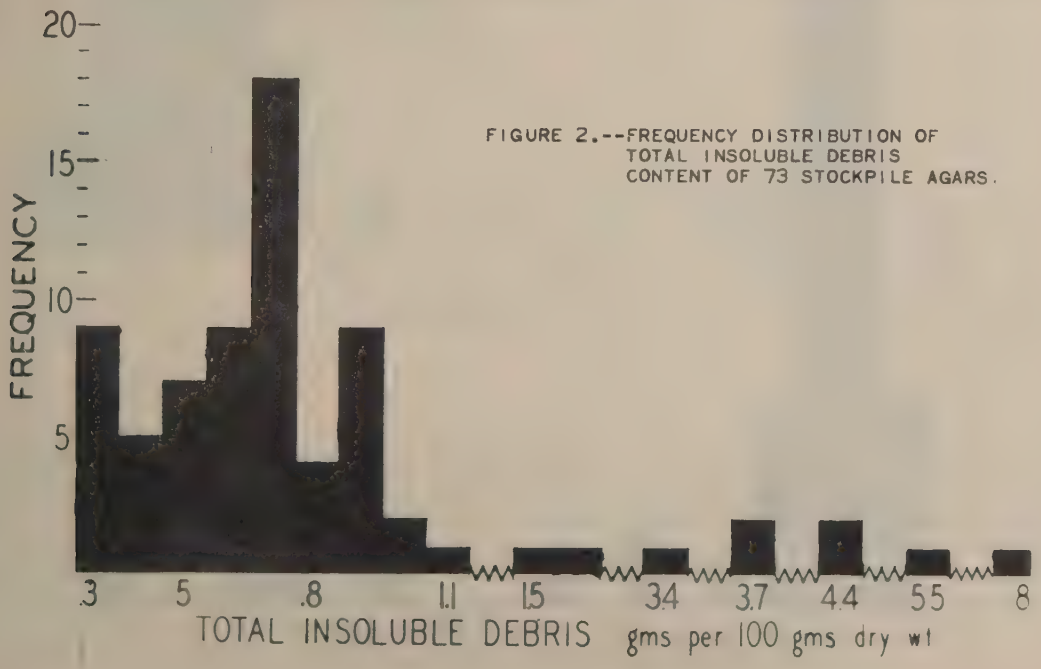

FIGURE 2.--FREQUENCY DISTRIBUTION OF TOTAL INSOLUBLE DEBRIS CONTENT OF 73 STOCKPILE AGARS.

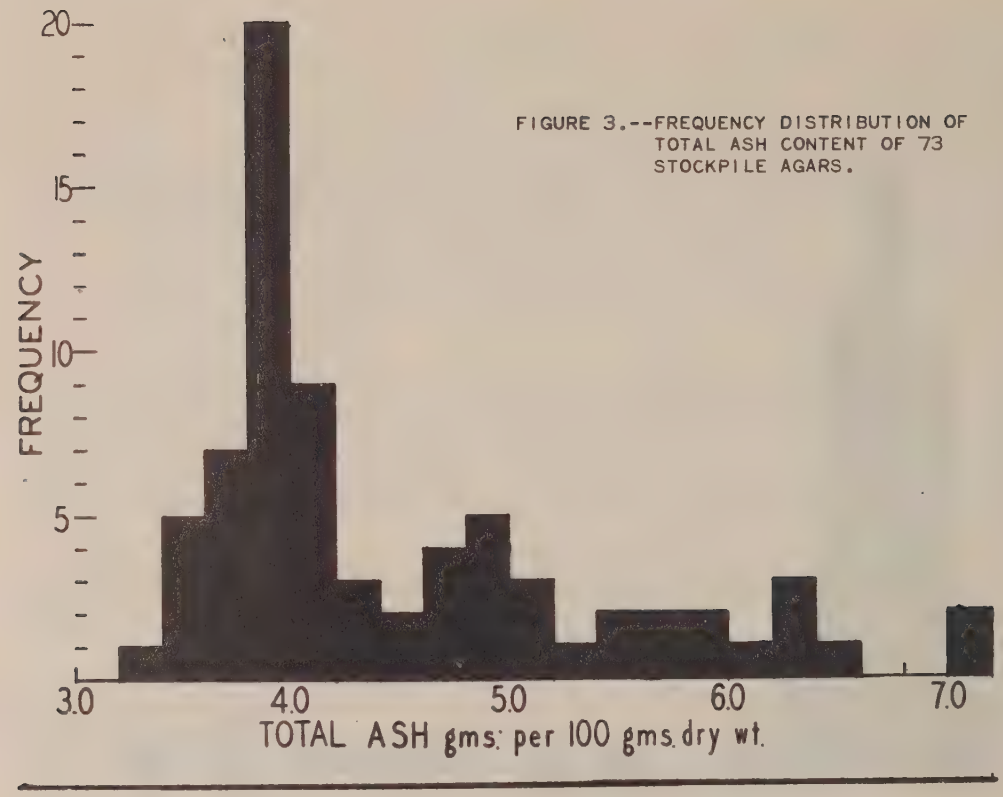

FIGURE 3.--FREQUENCY DISTRIBUTION OF TOTAL ASH CONTENT OF 73 STOCKPILE AGARS.

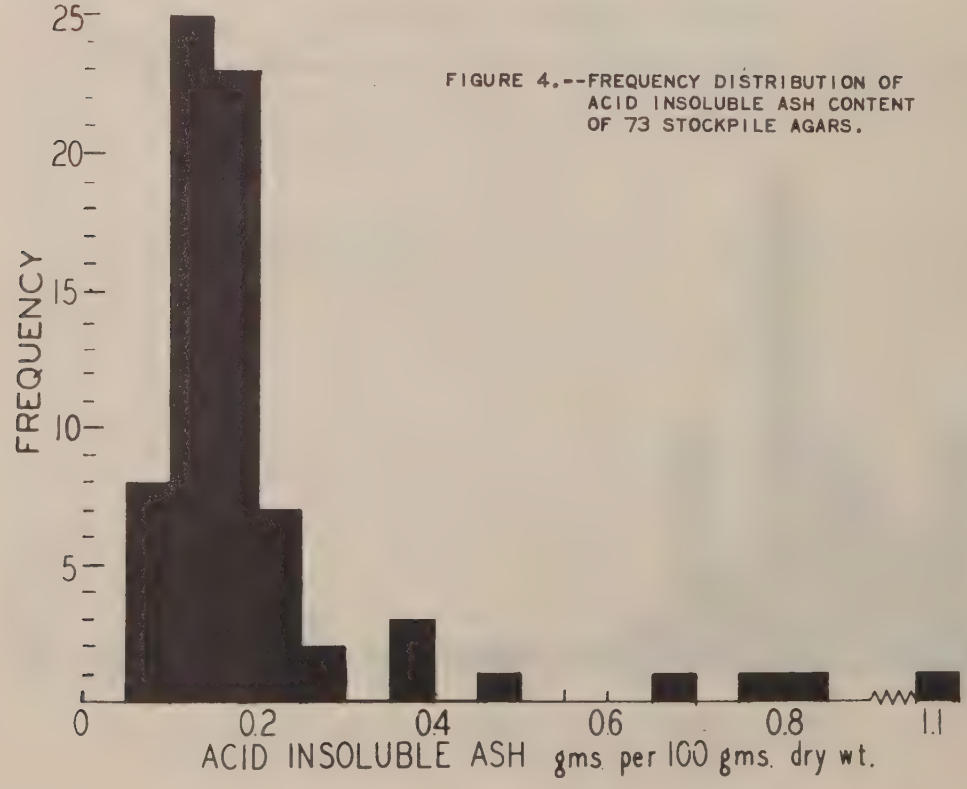

FIGURE 4.--FREQUENCY DISTRIBUTION OF ACID INSOLUBLE ASH CONTENT OF 73 STOCKPILE AGARS.

Acid insoluble ash.--The histogram for acid-insoluble ash content (Figure 4) shows a sharp frequency peak at 0.10 to 0.20 percent with 89 percent of the samples containing less than 0.25 percent. All the grade 1 samples of either Kobe or Yokahama are included in this group. The higher values, ranging up to 1.10 percent, are found exclusively among the grade 3 samples.

Iron.--The histogram (Figure 5) and data in Table 4 show that 88 percent of the samples have an iron content in the range from 30 to 90 mg. per kg. dry weight (parts per million). As with protein, insoluble debris, and acid insoluble ash, the iron content of all the grade 1 samples was within a limited range, the grade 3 samples showing a scattered frequency distribution up to a maximum of 348 parts per million of iron.

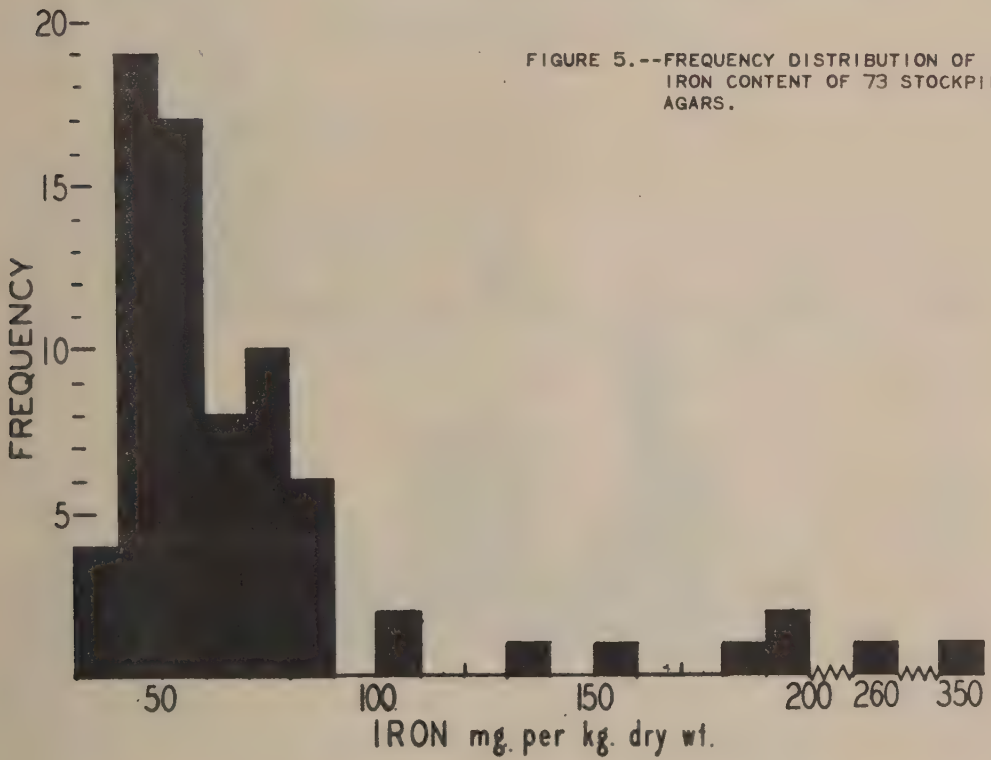

FIGURE 5.--FREQUENCY DISTRIBUTION OF IRON CONTENT OF 73 STOCKPILE AGARS.

Copper content as pictured in the histogram, Figure 6, did not have as sharp a distribution peak, and did not appear to be related to the grade of the agar. Fifty one (70 percent) of the samples contained from 4 to 8 parts per million, but with one exception the maximum copper content in any sample was less than 15 parts per million. Of 20 samples with more than 8 parts per million of copper, 16 are among the 62 grade 1 samples, the remaining 4 being found in the 11 grade 3 samples, giving every indication of truly random scatteration.

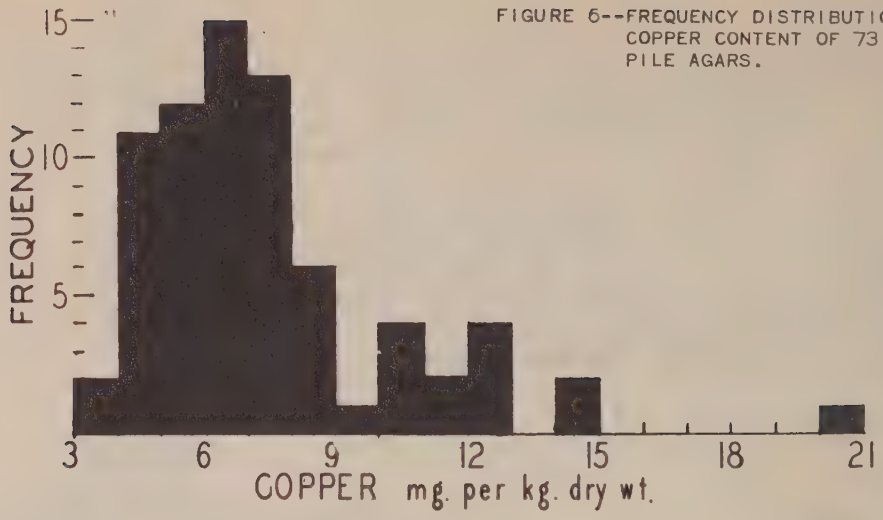

FIGURE 6--FREQUENCY DISTRIBUTION OF COPPER CONTENT OF 73 STOCKPILE AGARS.

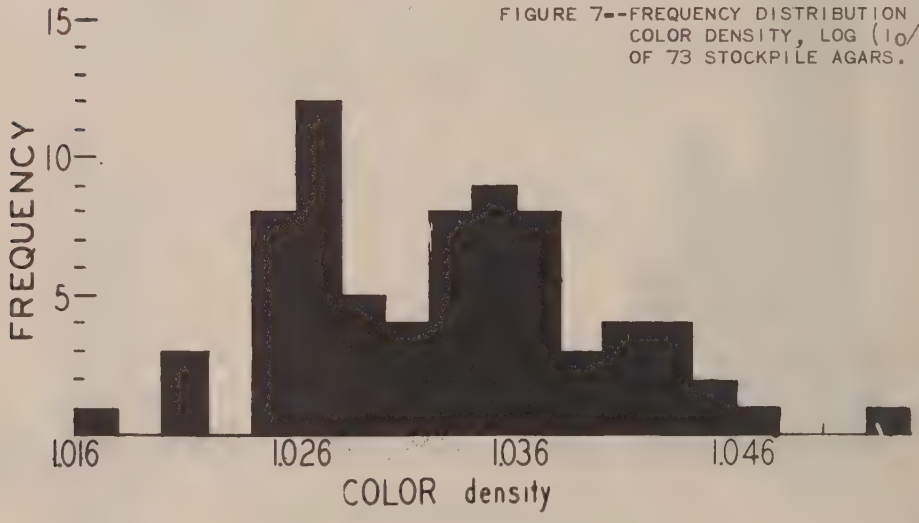

FIGURE 7--FREQUENCY DISTRIBUTION OF COLOR DENSITY, LOG (Io/I), OF 73 STOCKPILE AGARS.

Color.--The histogram showing the frequency distribution of color density values (Figure 7) shows no definite peak in the range 1.020 to 1.045. An examination of data in Table 4, indicates that although many of the grade 3 samples exhibit high color (density above 1.039) a very considerable number of the grade 1 samples were but slightly lighter colored, in the range of density from 1.034 to 1.037.

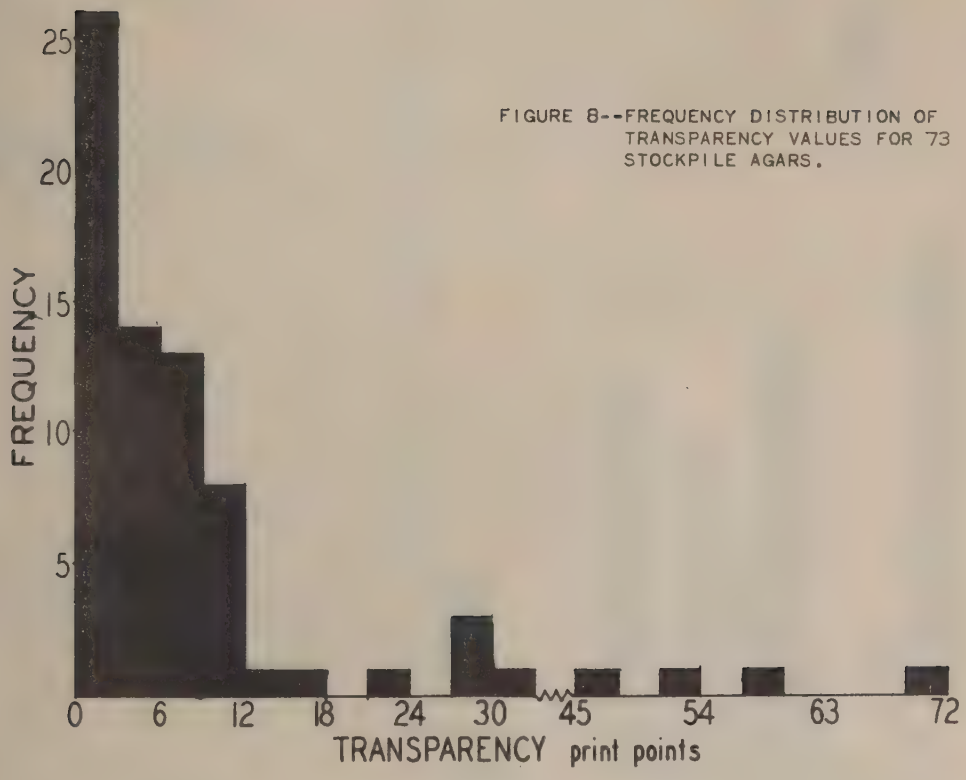

FIGURE 8--FREQUENCY DISTRIBUTION OF TRANSPARENCY VALUES FOR 73 STOCKPILE AGARS.

Transparency, as expressed in points size of print which can be read through 2 centimeters of gel shows a high frequency peak (Figure 8) at the smallest print, 2 to 4 type point, with the frequency decreasing rapidly to 12 point. Sixty-one samples (84 percent) of the agars produced gels of a transparency permitting the reading of 12 point or smaller print. Although most of the samples rated as relatively poor in this respect are grade 3, the correlation was not perfect as four grade 1 samples were among the 12 giving gels with a transparency represented by values greater than 12 point print.

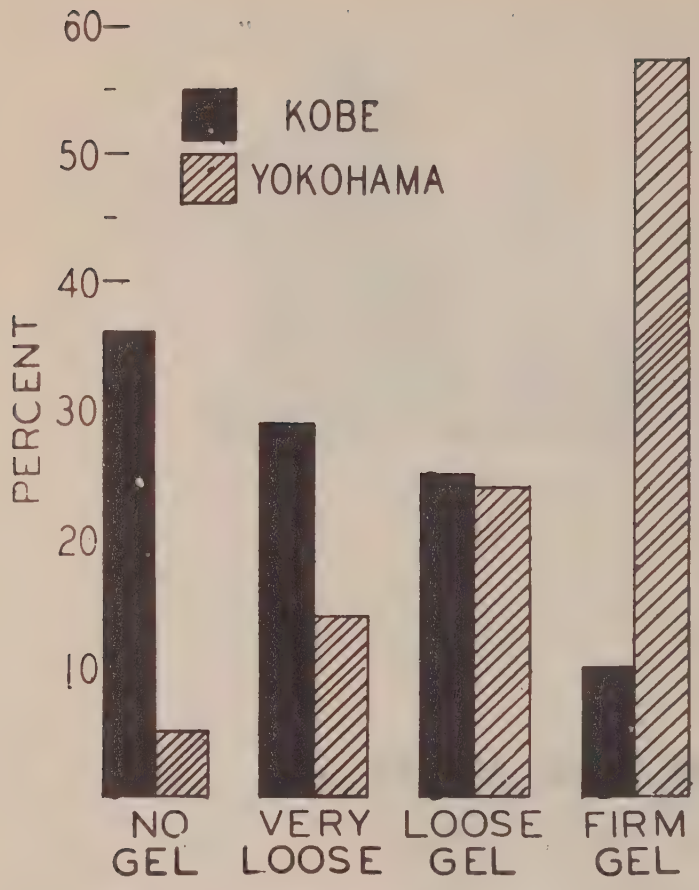

FIGURE 9--RELATIVE STRENGTH OF GEL FORMED AT 40°C., AS PERCENTAGE OF THE TOTAL NUMBERS OF SAMPLES FROM EACH LOCALITY, KOBE AND YOKAHAMA.

Temperature of gelation could not be depicted in the same manner as the other variables. None of the samples gelled at 45° and all gelled at 35°C. under test conditions. In Figure 9 are shown the percentages of the total number of samples from each source that formed firm, weak, very weak, or no gels in the 40°C. water bath, (3, 2, 1, and 0 respectively, in Table 4). When tabulated in this manner, the Kobe samples showed a definite trend toward the formation of weak gels or no gels at 40°C., while the Yokahama samples exhibited an even more marked trend to form firm gels at this temperature.

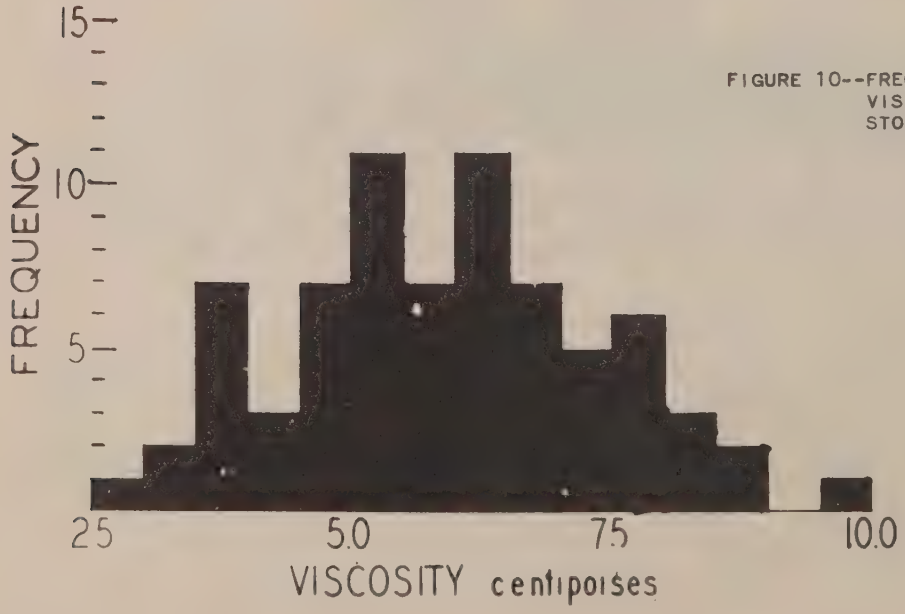

FIGURE 10--FREQUENCY DISTRIBUTION OF VISCOSITY VALUES FOR 73 STOCKPILE AGARS.

Viscosity for this series of samples shows the most uniform distribution of any of the variables studied (Figure 10). Values for all samples are between 2.5 and 10 centipoise with 64 samples (88 percent) in the range of viscosity from 3.5 to 8.0 centipoise. Distribution appears to be entirely random, with no relation to source or grade. In some instances, all samples from the same lot were fairly uniform, especially in lot 193 of the Yokahama grade 1, of which all 5 samples were low in viscosity, from 3.0 to 3.5 centipoise. However, in most cases, variation within a lot was about as large as variation in viscosity between different lots.

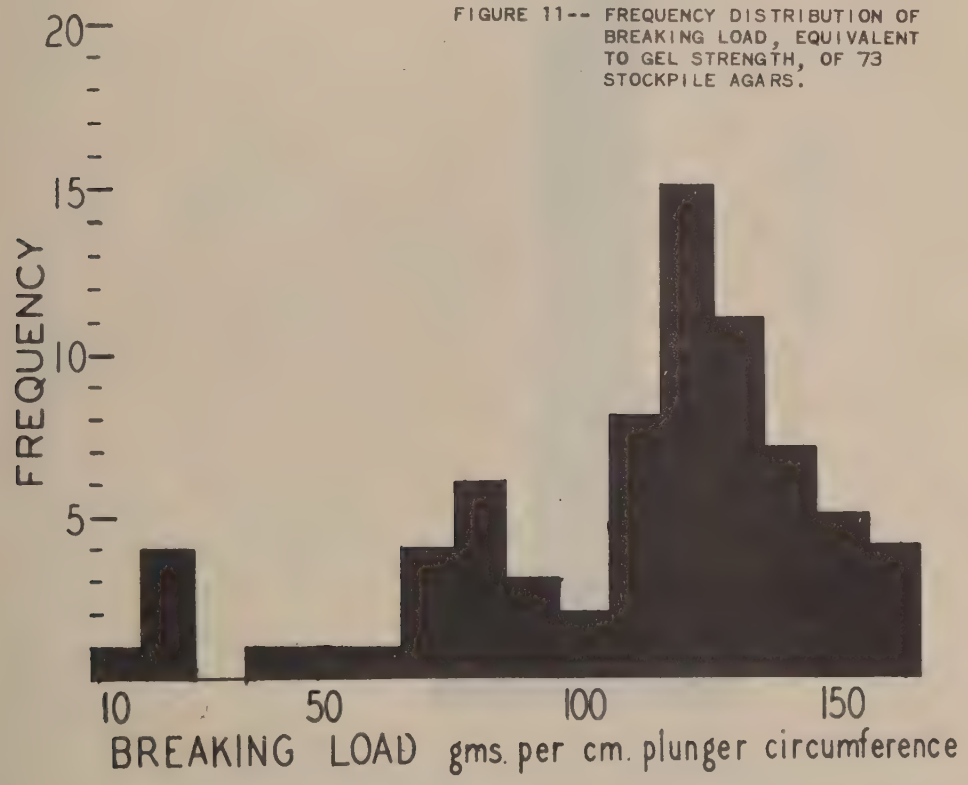

FIGURE 11-- FREQUENCY DISTRIBUTION OF BREAKING LOAD, EQUIVALENT TO GEL STRENGTH, OF 73 STOCKPILE AGARS.

Breaking load, expressed in grams per centimeter plunger circumference, and equivalent to gel strength, shows a rather random scatteration (Figure 11) although the histogram shows a small frequency peak at 80, and a higher peak at 120 gm. per cm. of plunger circumference. These peaks correspond roughly to the average breaking loads of the Yokahama and Kobe agar gels respectively. Although there were exceptions in each case, in general the Kobe gels had about 50 percent higher breaking strength than the Yokahama agar gels. There was no correlation between breaking load and grade of agar, though samples of the same lot gave gels of fairly uniform strength. The same lot beforementioned, No. 193 Yokahama grade 1, was unique in this respect with its five samples yielding gels with the lowest breaking loads of all 73 samples, namely 10 and 20 gm. per cm.

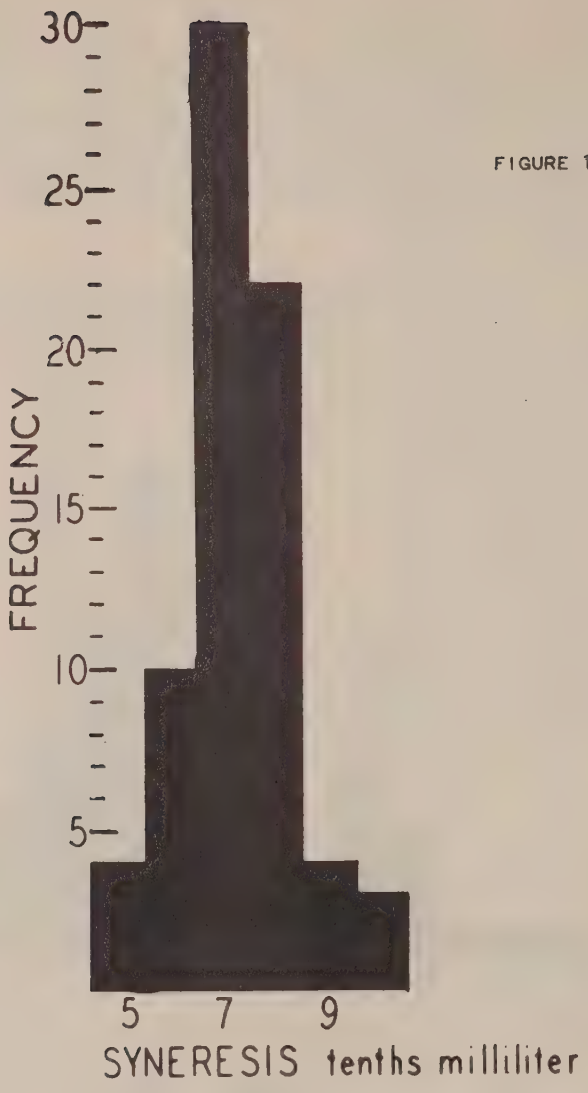

FIGURE 12--FREQUENCY DISTRIBUTION OF AMOUNT OF SYNERESIS DEVELOPED BY 73 STOCK-PILE AGARS.

Syneresis of all samples was in the limited range of 0.5 and 1.0 ml. expressed as free water per 100 gm. of gel. The histogram (Figure 12) shows a very high peak frequency at 0.8 ml., with syneresis of 0.9 ml. observed in almost as many sample gels. Distribution of the values for syneresis was entirely random with no relation to lot, grade or source.

TABLE 4--Chemical and physical

Trade designation and source Lot and sample designation	130 A	176 A	 B	 C	178 A	 B	 C	 D	 E	Kob
										Data calculated
Protein, percent	1.68	1.51	1.49	1.52	1.55	1.56	1.55	1.64	1.60	
Water insoluble debris, percent	0.9	0.5	0.6	0.4	0.3	0.6	0.3	0.3	0.3	
Total ash, percent	3.94	3.99	3.81	3.82	3.92	4.24	4.10	3.78	4.04	
Acid insoluble ash, percent	0.19	0.14	0.10	0.08	0.06	0.24	0.15	0.10	0.13	
Iron, parts per million	50	50	53	71	41	54	54	41	73	4
Copper, parts per million	10.2	6.8	5.9	20.1	6.4	4.4	5.8	7.1	5.6	1
										Data for 1.5 per
Color density, log. Io/I	1.028	1.030	1.028	1.031	1.035	1.036	1.033	1.031	1.037	
Transparency, type points	12	2	3	3	6	30	5	9	6	
Gelation at 40°C. 0=no gel to 3=firm gel	0	3	0	0	1	1	2	2	1	
Viscosity, centipoise	5.2	6.5	6.2	6.7	4.8	3.8	5.5	5.3	4.7	
Breaking load, gm. per cm. plunger circumference	130	120	120	130	120	130	110	140	120	16
Syneresis, ml. per 100 gm.	0.8	0.8	0.8	0.8	0.7	0.7	0.5	0.8	0.5	

TABLE 4--Chemical and physical c

Trade designation and source Lot and sample designation	186 A	 B	 C	187 A	190 A	 B	 A	 B	197 C	Kob
										Data calculated
Protein, percent	1.44	1.48	1.56	1.59	1.48	1.49	1.39	1.46	1.49	
Water insoluble debris, percent	0.4	0.6	0.9	0.3	0.4	1.0	0.3	0.9	0.9	
Total ash, percent	3.59	3.84	3.94	4.18	3.77	3.98	3.25	3.46	3.76	
Acid insoluble ash, percent	0.12	0.17	0.21	0.16	0.17	0.11	0.09	0.16	0.18	
Iron, parts per million	38	46	53	41	34	70	77	52	55	5
Copper, parts per million	5.5	7.5	6.2	5.0	7.0	14.8	7.8	3.9	4.4	
										Data for 1.5 per
Color density, log. Io/I	1.017	1.026	1.025	1.033	1.026	1.038	1.053	1.025	1.036	
Transparency, type points	2	6	9	2	3	7	11	9	12	
Gelation at 40°C. 0=no gel to 3=firm gel	1	1	1	0	0	3	0	1	2	
Viscosity, centipoise	6.8	6.0	6.3	5.5	7.6	3.8	2.6	5.3	5.2	
Breaking load, gm. per cm. plunger circumference	140	140	140	110	140	80	80	140	120	12
Syneresis, ml. per 100 gm.	0.7	0.8	0.6	0.7	0.7	0.8	0.7	0.7	0.9	

characteristics of stockpile agars

e Grade 1

		180		182						183			
F	G	A	B	A	B	C	D	E	F	A	B	C	D
to dry weight of agar													
1.64	1.49	1.62	1.64	1.63	1.72	1.57	1.52	1.48	1.55	1.68	1.48	1.74	1.69
0.4	0.3	0.8	0.7	0.5	0.7	0.7	0.7	0.9	0.5	0.6	0.3	0.6	0.7
3.95	3.85	3.55	3.70	4.44	3.89	4.63	3.88	3.50	3.41	3.90	4.12	4.19	4.04
0.14	0.10	0.09	0.08	0.15	0.18	0.12	0.19	0.15	0.11	0.20	0.13	0.19	0.19
7	33	47	80	85	41	46	54	50	43	86	55	48	41
8.5	7.5	4.7	12.1	11.2	6.3	5.8	6.1	8.3	7.3	7.0	6.4	6.1	6.4
cent (dry weight) gels													
1.028	1.028	1.027	1.036	1.033	1.037	1.036	1.032	1.022	1.025	1.035	1.041	1.037	1.037
6	4	3	5	4	18	9	14	11	2	10	4	12	9
1	2	1	0	2	1	3	0	1	0	3	1	0	2
6.5	6.8	6.5	4.9	7.8	3.8	7.7	3.6	5.4	5.3	4.8	5.4	5.7	5.5
0	160	130	110	130	120	120	100	130	120	130	120	120	140
0.7	0.8	0.7	1.0	0.7	0.6	0.8	0.7	0.8	0.8	0.8	0.6	0.6	0.8

haracteristics of stockpile agars, cont'd

e Grade 1

		198			200		201				
D	E	A	B	C	A	B	A	B	C	D	E
to dry weight of agar											
1.39	1.43	1.56	1.48	1.61	1.56	1.59	1.83	1.59	1.49	1.51	1.60
1.0	0.7	0.5	0.6	0.4	0.5	0.5	0.7	0.9	0.9	0.8	0.7
3.61	3.83	4.26	3.82	4.29	4.04	4.14	3.93	3.73	3.94	3.99	3.96
0.27	0.22	0.16	0.13	0.18	0.12	0.11	0.22	0.13	0.17	0.17	0.09
5	45	55	45	47	36	70	46	44	50	42	53
7.4	8.7	4.4	7.2	4.5	5.0	9.5	4.6	6.9	6.4	5.8	8.7
cent (dry weight) gels											
.033	1.027	1.027	1.027	1.029	1.028	1.037	1.033	1.041	1.039	1.029	1.035
	10	2	2	2	3	3	9	3	10	8	3
	1	0	0	0	0	1	0	0	0	0	2
.9	4.7	5.8	5.0	6.0	5.7	4,2	7.0	5.0	7.7	7.7	6.7
	120	120	110	110	120	90	160	130	150	150	130
.7	0.7	0.9	0.7	0.8	0.7	0.7	0.8	0.7	1.0	0.6	1.0

Trade designation and source	Kobe Grade 3								Yokahama Grade 1								
Lot and sample designation	183			199		201			175				193				
	A	B	C	A	B	A	B	C	A	B	C	D	A	B	C	D	E
	Data calculated to dry weight of agar								Data calculated to dry weight of agar								
Protein, percent	3.06	3.10	1.51	2.60	3.53	4.16	2.83	1.76	1.45	1.48	1.55	1.48	1.68	1.74	1.68	1.64	1.71
Water insoluble debris, percent	4.3	4.4	0.3	3.7	5.5	7.8	3.7	0.7	0.7	0.9	0.9	0.8	0.7	0.6	0.6	0.7	0.8
Total ash, percent	4.81	5.61	3.86	4.87	5.41	6.31	4.81	6.58	5.04	5.08	5.05	5.22	5.99	6.08	6.37	5.86	6.37
Acid insoluble ash, percent	0.52	0.78	0.14	0.65	1.13	0.80	0.37	0.15	0.14	0.14	0.15	0.12	0.22	0.16	0.18	0.13	0.21
Iron, parts per million	188	194	49	261	348	194	108	55	65	67	76	83	73	64	64	75	77
Copper, parts per million	8.8	6.9	4.8	7.7	7.4	10.1	11.7	4.6	6.3	5.4	5.8	5.1	4.9	10.3	8.5	8.9	6.9
	Data for 1.5 percent (dry weight) gels								Data for 1.5 percent (dry weight) gels								
Color density, log. Io/I	1.043	1.043	1.028	1.033	1.037	1.045	1.038	1.022	1.034	1.033	1.030	1.041	1.036	1.047	1.040	1.044	1.045
Transparency, type points	30	24	3	60	72	72	48	2	2	1	2	2	6	6	6	9	7
Gelation at 40°C. Omo gel to 3-firm gel	2	2	2	1	2	2	2	3	3	3	3	3	2	3	2	2	2
Viscosity, centipoise	6.1	6.4	6.2	4.4	5.7	6.1	9.6	8.1	7.5	7.3	7.3	7.3	3.5	3.1	3.5	3.0	3.5
Breaking load, gm. per cm. plunger circumference	130	130	150	100	130	90	160	70	110	90	110	80	20	10	20	20	20
Syneresis, ml. per 100 gm.	0.6	0.8	0.7	0.7	0.8	0.9	0.7	0.7	0.8	0.8	0.8	0.8	0.7	0.8	0.6	0.6	0.5

TABLE 4--Chemical and physical characteristics of stockpile agars, cont'd

Trade designation and source	Yokahama Grade 1, cont'd									Yokahama Grade 3		
Lot and sample designation	196	197		201						201		
	A	A	B	A	B	C	D	E	F	A	B	C
				Data calculated to dry weight of agar								
Protein, percent	1.35	1.50	1.60	1.60	1.64	1.50	1.54	1.52	1.53	2.14	2.92	3.39
Water insoluble debris, percent	0.7	0.6	0.7	0.7	0.5	1.1	0.7	0.7	0.7	1.6	3.4	1.5
Total ash, percent	3.62	4.78	4.56	5.48	7.09	4.07	4.89	4.81	4.69	5.74	4.60	7.07
Acid insoluble ash, percent	0.17	0.11	0.09	0.13	0.11	0.19	0.12	0.11	0.09	0.26	0.36	0.38
Iron, parts per million	44	69	69	137	80	51	69	65	73	105	87	152
Copper, parts per million	4.4	14.4	5.2	4.8	12.4	3.9	10.1	7.8	7.8	6.3	12.1	6.0
				Data for 1.5 percent (dry weight) gels								
Color density, log. Io/I	1.039	1.031	1.026	1.027	1.035	1.029	1.021	1.026	1.025	1.044	1.038	1.042
Transparency, type points	3	3	6	4	2	33	2	4	2	8	54	30
Gelation at 40°C. Omo gel to 3-firm gel	0	2	3	3	3	1	3	3	3	3	3	3
Viscosity, centipoise	4.3	5.1	5.0	4.7	6.0	7.4	8.1	8.3	8.8	6.2	8.5	6.2
Breaking load, gm. per cm. plunger circumference	110	70	50	40	60	150	80	70	80	80	150	70
Syneresis, ml. per 100 gm.	0.7	0.7	0.5	0.7	0.6	0.7	0.7	0.6	0.7	0.7	0.9	0.7

Discussion

In general, agar is a gum extracted from seaweeds belonging to the class Rhodophyceae. In chemical constitution, it is the sulfuric acid ester of a galacten. Because of this constitution, the ash content is indicative of molecular size. In the series examined, there is a definite indication of a relation between acid soluble ash content and the source of agar. The Kobe grade 1 samples averaged 3.76 percent acid soluble ash compared to the average of the Yokahama grade 1 samples of 5.16 percent. Even including the Kobe grade 3 samples (with two abnormally high values) the average is increased to only 3.91 percent acid soluble ash as compared with 5.20 percent for all the Yokahama agars. Apparently there is a source difference, probably in the species of seaweed making up the bulk of raw stock in the two localities. With the total ash content influenced by both molecular ash and inorganic impurities, it is difficult to establish an entirely satisfactory maximum limit. These data show that the 6.5 percent level would include almost all the agars in this group, excepting a very few grade 3 samples, and this limit would exclude most of the unsatisfactory gums with smaller molecule size. Certain highly purified extracts from Gracilaria, for example, could meet this limit, but are readily differentiated on basis of viscosity or other physical properties.

As mentioned in the summary of results, the average breaking load of the agars from the two localities differs also, resulting in two frequency peaks in the histogram (Figure 11). The values in this case are little influenced by grade, the Kobe agars averaging 124 gm. per cm. compared to the average breaking load of the Yokahama agars of 66 gm. per cm. This latter value is unduly influenced by the five weak agars of lot 193. If these values are omitted, the average for the Yokahama agars is 87 gm. per cm. There is then a definite relation of breaking load to source which in this series of samples has already been related to the size of the agar molecule, and the amount of acid soluble ash. The numerical relation is inverse; as the percent of ash increases, the strength decreases. The correlation coefficient of these two variables was found to be $r = -.745$, a highly significant value. The regression line is represented by: Breaking load, (gm. per cm.) (B) = 242.4 - 31.2 x percent acid soluble ash, and gives a fairly good estimate of the expected gel strength, except for extreme values.

A third property of agar which appears to be related to the source of the agar in this series, and therefore to the amount of acid soluble ash, breaking load and molecular size, is the degree of gelation at 40°C. A considerable difference in the state of gelation was observed, with the Kobe (large molecule) agars exhibiting a lower gel point than the Yokahama samples (Figure 9).

These marked indications of variation of structural and physical characteristics with source, and the data of Table 2 showing the variations that are possible for some species of Rhodophyceae, make it necessary that "agar" in the restricted bacteriological sense be defined on the basis of physical characteristics desired. The limits of these characteristics which are acceptable vary with the individual bacteriologist and the particular use for which

the agar is intended. The following are suggested as practical limits: Breaking load not less than 50 gm. per cm.; syneresis, not more than 1.2 ml.; viscosity, not greater than 12 centipoise; color density, no greater than 1.048; transparency, to permit the reading of 12 point type through 2 cm. of gel. These limits are sufficiently broad to include all but a few samples having abnormal gel strength, color or transparency, yet are restrictive enough to give a uniform working material.

Another physical limitation that was met by all the commercial samples but not by many other seaweed extractives is that the sol should be liquid at 45° and set to a firm gel at 35°C.

One important physical characteristic of agar which was mentioned in the introduction and which constitutes one of its principle attributes, is that the gels it forms are thermally reversible, melting at a substantially higher temperature than that at which they set. This property is characteristic of all the gums extracted from Rhodophyceae but nevertheless should be a fundamental part of any definition of agar.

Another desirable property of the gums extracted from Rhodophyceae is their ability to form gels in the pH range most used by bacteriologists. They should not be affected in too great an extent in this pH range by the ordinary procedures for sterilizing media. The provision that gels conform to the standards set after autoclaving under normal conditions at any pH value in the range 6.0 to 8.0 would be a necessary limitation for bacteriological agar.

In addition to these physical properties, which are, in several cases, highly characteristic of the true Gelidium-agar, certain of the chemical properties are valuable primarily as indications of the quality of agar. Four tests: Protein (N x 6.25), water-insoluble debris, acid insoluble ash, and iron, show correlation as to grade to the extent that maximum limits could be established to determine grade 1 agar. Determined on a dry basis, these maximum limits would be as follows: 1.90 percent protein (N x 6.25), 1.0 percent water-insoluble debris, 0.25 percent acid insoluble ash, and 90 parts per million of iron.

Copper was found in small quantities in all the samples tested. None of the values were abnormally high and the analysis for copper does not appear of value as an indication of contamination or source differences. The determination of copper might be desirable in certain circumstances of special use, such as work with organisms highly sensitive to metallic ions or tests of similar nature.

All the above criteria can only result in obtaining a clean agar having suitable physical characteristics. Whether these are sufficient to obtain an agar suitable for bacteriological use can be determined by practical application. This work constitutes section II of the paper.

Conclusions

Total ash or more exactly acid soluble ash content, breaking load and degree of gelation of the agar sol at 40°C., are all interrelated and appear to be linked with the size of the agar molecule. In this series these variables were also related to the source of the seaweed, Kobe or Yokahama. No proof is available but evidence points to a difference in species of weed used as raw material in the different locations of origin.

Since it is mainly in the physical properties of agar that the bacteriologist is interested and since wide variations in these properties are possible, practical limits of definition for breaking load, syneresis, viscosity, color density and transparency are suggested. Thermal reversibility of the gel, and stability in the pH range 6.0 to 8.0 should also be considered in any definition of agar.

Determination of protein, total water insoluble debris, acid insoluble ash and iron are all valuable tests for indicating the cleanliness or quality of agar. Tolerances could be readily established which would exclude agars containing undesirable amounts of contaminating impurities. Determination of copper content appears to be unnecessary except for special purposes.

Section II of the paper will determine by practical application whether the above criteria are sufficient to define agar for bacteriological use.

II. BACTERIOLOGICAL STUDIES

Introduction

When all bacteriological manipulations are considered, a large number of requisite properties of agar are indicated. Agar must form a true gel in dilute solution. Gelation should occur within a narrow range of temperature. Solutions of agar must not be too viscous or contain too much inorganic material. Syneresis cannot be excessive and the set gel should adhere to the surface of the petri dish. The gel ought to be clear, possess a satisfactory degree of firmness, have at least moderate strength and should be thermally reversible. The desirable properties of the gel should not be affected by the range in pH or the methods of sterilization encountered in bacteriological practice. There are also a number of purely bacteriological characteristics which require consideration in the establishment of tolerances. An ideal agar preparation is free of materials which may interfere with morphological, cultural or antigenic properties of bacteria, and these are not ordinarily apparent from chemical tests. Few seaweed extracts possess all of these desirable qualities.

This section of the paper deals mainly with bacteriological tests. These include the influence of agar *per se* on standard plate counts, on colony size, and other colonial characteristics. The findings have been correlated with the physical and chemical properties whenever this has been possible.

Materials

Two groups of agar samples were used in this study. The first consisted of seven agar samples considered representative of all types available, received from the Difco Laboratories. The physical and chemical properties of these agars are indicated by the data in Table 5. This group was studied in detail from a bacteriological viewpoint. The second group consisted of the 73 agar samples obtained from the government stockpile through the courtesy of the War Production Board and the Difco Laboratories. These were not studied as extensively as Group 1, bacteriologically. As an example of other gum types, extracts from Gracilaria confervoides (North Carolina) also were studied.

TABLE 5. Physical and chemical characteristics of special agars

Analyses	Agar Samples A	B	C	D	E	F	G
Moisture, percent	17.8	16.2	14.0	14.5	14.9	17.2	14.6
Data calculated to dry weight							
Protein (Nx6.25), percent	1.03	0.50	0.90	0.70	0.95	1.56	1.14
Total insoluble debris, percent	0.1	0.0	0.1	0.6	1.1	0.7	0.8
Total ash, percent	5.03	3.13	3.87	3.77	4.34	4.82	3.78
Acid insoluble ash, percent	0.12	0.15	0.09	0.16	0.40	0.18	0.38
Iron, micrograms per gram	62	53	35	136	101	51	292
Copper, micrograms per gram	7.8	6.9	7.5	12.5	16.2	7.0	38.0
Data for 1.5 percent (dry weight) gels							
Color density, log. Io/I	1.026	1.025	1.055	1.024	1.024	1.040	1.020
Breaking load gm. per cm. of plunger circumference	150	180	90	90	110	80	150
Viscosity at 45°C., centipoise	7	11	3	6	5	5	3
Syneresis, ml. per 100 gm.	0.7	0.8	0.7	0.7	0.8	0.8	0.8

Methods

Preparation of culture media. The general method employed for making the culture media was to prepare a large batch of broth base, and to this the various agars were added separately in flasks. Whenever possible, a single lot of dehydrated broth medium (Bacto) was employed to insure uniformity throughout the experiments. When this was not feasible, as in the case of the Standard Methods agar for milk plate counts, a large quantity of base was prepared, the respective

agars added and then Tryptone Glucose Extract Agar (Bacto) was used as a control medium. Throughout all of the experiments, agar was used in a concentration of 1.5 percent.

In all cases the agar was dissolved by heating in an Arnold steamer. The solutions were filtered through absorbent cotton except for the Gracilaria extracts which had to be filtered through glass wool. Sterilization was accomplished by autoclaving at 15 lbs. pressure for 15 min.

For pure culture studies, dehydrated Nutrient Broth (Bacto) was employed as a base. When blood agar was prepared, 0.85 sodium chloride was added to the base, and one percent rabbit blood was added just prior to pouring of the plates.

Milk plate counts were made with the medium recommended by the Committee on Standard Methods for Milk Analysis. The pH was adjusted prior to the addition of the agar in order to maintain constancy in all respects except for the agar.

Plate counts. Comparative plate counts were made with both groups of agars on each of a number of samples of standard pasteurized milk. The medium was cooled to 45°C. before pouring, and the plates were incubated at 37°C. for 48 hours. In the case of Gracilaria it was necessary to pour plates with the medium at 60°C. since the gel set very rapidly below this temperature.

Colony size. The size of colonies on the various agar media was determined by two methods. In the first, where colonies were sufficiently large, photograms were made of the plates and the size of 10 representative colonies was measured directly. The image of pin-point colonies was projected on a ground-glass viewing screen at a 10 x magnification. The outline of 10 individual colonies was then traced on paper, and the diameter was measured with dividers.

Hemolysis. Hemolysis was determined by using whole rabbit blood. Where the red blood cells were hemolyzed by the agar medium alone, no cultural studies were made. In all other cases, however, strains of alpha and beta hemolytic streptococci and staphylococci were employed in pour and streak plates.

Cultural and colonial characteristics. Test organisms were used to determine whether the agar per se would alter either their cultural or colonial characteristics. Known strains of Escherichia, Aerobacter, Staphylococcus, Streptococcus and Neisseria were transferred serially on suitable media prepared with the test agars. After 10 successive serial transfers, the major cultural characteristics were compared with those determined initially.

Colonial characteristics on blood agar, eosin methylene blue agar, nutrient agar and motility test medium were also determined with the test agars and compared with the characteristics of the organisms grown on the corresponding media with Bacto agar. Particular attention was paid to the possible S-R variations which might occur. The motility test medium was used to observe the effect of the gel consistency on the reliability of the test with different agars.

Supplementary tests. This section deals with bacteriological tests primarily. However, several simple physical tests were made to determine their value for judging the suitability of agar for bacteriological use. The only one reported is that for gel texture.

The degree of firmness of the gel was determined by rubbing a finger over the surface. The judges gave a numerical rating to their estimate, ranging from 1 for a very weak gel to 5 for a firm textured gel.

Results

Plate counts. When milk was used as an inoculum, there were no consistent differences in the colony counts obtained with the various agars. The data on the seven test agars are shown in Table 6. The colony counts are based on an average of 60 poured plates, using 20 different milk samples. The variations in counts are not consistent and probably represent variability in sampling rather than in the agar constituents.

TABLE 6. Data on colony count, and percent plates with spreaders with twenty samples of pasteurized milk plated in triplicate

Medium	Inoculum, milliliter	Agar Samples							
		Bacto	A	B	C	D	E	F	G
		Average colony counts for twenty milk samples							
TGEA	1.0	136	77	47	2/	117	212	65	122
"	0.1	96	66	49	41	34	35	50	53
"	0.01	5	9	6	9	6	6	12	4
NA	1.0	141	158	107	127	122	130	181	142
"	0.1	77	82	69	71	68	71	73	78
"	0.01	11	9	7	9	7	8	10	8
		Percent of plates with spreaders							
TGEA 1/	1.0	56	25	50	60	25	29	12	29
" 1/	0.1	5	5	0	5	5	0	0	1
" 1/	0.01	0	0	0	0	0	0	0	0
NA 1/	1.0	3	4	8	16	0	8	4	30
" 1/	0.1	0	0	3	0	0	0	3	20
" 1/	0.01	0	0	0	0	0	0	3	6

TGEA equals tryptone glucose extract agar, and
NA equals nutrient agar.
1/ Plates showing spreaders covering more than half of the surface.
2/ Either covered with spreaders, or too many colonies to count.

The number of spreaders appears to be correlated with the amount of inoculum rather than with the agar per se. The distribution of spreaders throughout the entire series indicates that there is probably no correlation between the incidence of spreaders and any physical or chemical properties of the agars.

TABLE 7. Data on plate count of pasteurized milk inoculated on stockpile agars in tryptone glucose extract base

Stockpile sample designation	Average plate count	Colonies over 1 millimeter in diameter	Stockpile sample designation	Average plate count	Colonies over 1 millimeter in diameter	Stockpile sample designation	Average plate count	Colonies over 1 millimeter in diameter
		Percent			Percent			Percent
K1-180B	404	71	K1-197E	149	24	Y1-175B	161	16
K1-197A	314	71	Y1-197B	193	24	K1-186B	191	15
K1-201B	221	48	Y1-175A	176	24	Y1-197A	178	15
Y1-201F	204	42	K1-201D	149	24	K1-197D	265	15
K1-186C	226	40	K1-178G	299	23	K1-176C	186	14
K1-190B	209	36	K1-182F	268	23	K1-198A	139	14
K1-130A	245	33	K3-199A	213	23	K1-200A	95	14
K3-201A	337	33	K1-190A	182	22	K3-201B	168	12
Y1-193C	233	33	K1-186A	298	20	K1-187A	170	12
Y1-193B	192	31	Y1-201B	151	20	K1-180A	255	12
K1-183B	178	29	K1-182A	208	20	Y1-201C	145	11
K1-183C	199	28	K1-182D	210	20	K3-183A	145	11
K1-182B	169	28	K1-198C	216	20	Y1-196A	189	11
K1-178D	202	28	K1-197B	111	20	K1-178A	164	10
K1-175C	169	28	K3-183B	170	19	K3-199B	116	9
K1-176B	186	28	Y1-175D	142	18	K3-201C	119	9
K1-182E	188	28	Y1-201E	163	18	K3-183C	132	9
Y1-201A	167	27	K1-198B	196	18	K1-178F	160	9
Y1-193D	230	26	Y3-201C	157	18	K1-200B	133	7
K1-201C	139	26	K1-178B	192	17	Y3-201B	215	7
Y1-201D	210	25	K1-176A	105	17	K1-178E	149	6
K1-201A	312	24	K1-182C	113	16	K1-201E	147	5
K1-183D	298	24	Y3-201A	199	16	K1-197C	127	5
						K1-178C	143	5

For sample identification refer to Table No. 4.
Coefficient of correlation of average plate count to percent colonies over 1 mm. = 0.568 which is highly significant.

A plating experiment was run with the 73 agar samples from the government stockpile using the Tryptone Glucose Extract base. A single sample of pasteurized milk diluted 1 to 10 was used as the inoculum for the entire series. Plates were poured in triplicate and the colony counts averaged for each agar sample (Table 7). The counts on the plates made from individual agars varied from 95 to 400 colonies. There was no apparent correlation between the number of colonies and the source of the agar or its physical and chemical properties.

It was observed that some of the government stockpile agars yielded greater numbers of pin-point colonies than others. Separate counts were made on the number of colonies one millimeter in diameter and over, and the percentage of these larger colonies was calculated. These data are also presented in

Table 7. The percentage of large colonies varied from 5 to 71 and shows a very significant relation to the average plate count, the greater percentage of large colonies tending to go with the higher counts. There did not appear to be any influence of the source of agar or the physical and chemical properties on colony size (Table 4). The only reasonable explanation for these differences seemed to be the possible presence of undetermined nutritive factors in the various agars.

To test this possibility a Tryptone Glucose Extract medium was prepared to which was added 0.5 percent liver extract (fraction L, Wilson). Although the control medium to which no liver extract was added yielded an average colony count of 226 per 0.1 ml. of pasteurized milk, the agar media prepared from the seven test agars plus the liver extract, had such an excess of colonies and overgrowth that the plates were not countable. It was not practicable to carry this procedure through with the lot of 73 agars nor was the lot of 7 checked with a smaller inoculum. The indication is that, if the nutritive level of the media is raised near an optimum by the addition of liver extract, the variability in counts due to unknown factors in the agars can be greatly reduced.

In order to determine some of the nutritive factors which might be responsible for this increased count, samples of diluted pasteurized milk were plated on Tryptone Glucose Extract Agar. To separate replicate samples were added a single vitamin or a mixture of the four vitamins. The quantities of the single vitamins were as follows: 1 microgram of thiamine, 1.5 micrograms of riboflavin, 0.75 microgram of calcium pantothenate and 1.5 micrograms of nicotinic acid per 100 ml. of medium. The mixture contained one-fourth of these amounts of each vitamin. The average data from three experiments are presented in Table 8.

The data indicate that the colony counts can be increased from 75 to 100 percent by the addition of individual vitamins to the culture medium. On the other hand, a mixture of all four vitamins yielded colony counts that were lower than those obtained with the individual compounds and but little higher than the control of group. The percentage of colonies over 1 mm. was increased in all cases, indicating that the addition of the nutritive factors to the medium increases the average colony size. Thus it appears that plate counts and colony size depend to some degree upon the nutritive qualities of agar *per se* in the case of the standard medium for milk plate counts.

Eighteen samples of commercial pasteurized milk were plated simultaneously on media prepared with *Gracilaria* extract and agar, and the colony counts compared. In all cases fewer colonies grew on the *Gracilaria* than on the agar medium. An average of the counts indicated that the number of colonies appearing on the *Gracilaria* gels amounted to only 41 percent of those on the agar.

Untreated surface water samples were also plated on *Gracilaria* and agar media. On the average, the counts obtained on the *Gracilaria* gel were only 9 percent of those appearing on the agar (see Table 9). This is based on triplicate platings with each sample on each medium.

TABLE 8. Colony count and size when pasteurized milk was inoculated in tryptone glucose extract agar containing certain added vitamins

Medium	Supplement micrograms per 100 ml. of medium	Agar Samples A	B	C	D	E	F	G	Mean	Standard error
		Plate Counts								
Control	-	145	186	150	115	137	127	171	147	9.3
Thiamine	1.0	214	217	220	242	219	221	218	222	3.5
Riboflavin	1.5	207	226	291	267	226	239	341	257	17.63
Calcium Pantothenate	0.75	289	259	261	275	270	308	314	282	8.3
Nicotinic acid	1.5	238	264	228	266	301	307	338	277	14.96
Mixture	1/	183	131	146	184	191	220	197	179	11.53
		Percent colonies over 1 millimeter in size								
Control	-	19	23	31	16	18	22	14	20	
Thiamine	1.0	48	56	40	33	40	42	24	40	
Riboflavin	1.5	42	42	33	30	37	33	20	34	
Calcium Pantothenate	0.75	38	34	36	27	33	36	21	32	
Nicotinic acid	1.5	53	59	33	32	36	32	23	38	
Mixture	1/	43	53	37	43	38	30	20	38	

1/ Thiamine, 0.25; riboflavin, 0.37; calcium pantothenate, 0.19 and nicotinic acid, 0.37 micrograms per 100 ml. of medium.

TABLE 9. Plate counts with Gracilaria and agar media when inoculated with surface water

Water sample	Plate count per ml. of water		Ratio of count on Gracilaria to count on agar
	Gracilaria	Agar	
1	150	4400	0.03
2	75	150	0.50
3	500	2900	0.17
4	65	1050	0.06
5	2500	18000	0.14
6	130	13500	0.09
7	475	2670	0.18
8	60	500	0.12

Aside from the low counts obtained with the Gracilaria media, there were other objections to the use of this gel. The amount of water of syneresis was so great that it formed puddles on the surface of the gel. Because of this excessive moisture, the medium would not adhere to the surface of the culture dish. The high gelling temperature not only reduced the colony counts but also made the distribution of the inoculum difficult because the sol would set as soon as it made contact with the petri dish. These undesirable properties made Gracilaria extracts unsuitable for plate count work.

TABLE 10. Effect of crowding and of differences in the agar base, on the colony size of Escherichia coli, grown with nutrient broth

Average diameter in millimeters of ten representative colonies, on high count and low count plates

Stockpile sample no.	High	Low	Stockpile sample no.	High	Low	Stockpile sample no.	High	Low
K1-130A	3.8	7.5	K1-187A	2.2	8.6	Y1-175A	4.4	9.3
K1-176A	3.7	7.4	K1-190A	4.4	5.2	Y1-175B	4.2	6.0
K1-176B	8.3	-	K1-190B	2.4	5.7	Y1-175C	3.2	3.8
K1-176C	-	4.6	K1-197A	1.9	7.6	Y1-175D	1.3	8.3
K1-178A	2.4	7.4	K1-197B	2.5	3.4	Y1-193A	3.8	9.6
K1-178B	3.0	8.3	K1-197C	3.6	10.4	Y1-193B	1.7	-
K1-178C	3.2	4.8	K1-197D	3.4	9.1	Y1-193C	3.2	11.7
K1-178D	3.4	11.9	K1-197E	3.6	11.6	Y1-193D	2.7	6.4
K1-178E	3.9	12.6	K1-198A	3.5	12.9	Y1-193E	3.7	4.8
K1-178F	6.3	9.5	K1-198B	2.9	9.8	Y1-196A	1.7	15.0
K1-178G	4.3	8.7	K1-198C	3.6	4.0	Y1-197A	3.0	10.2
K1-180A	2.2	6.2	K1-200A	1.9	10.1	Y1-197B	3.1	8.7
K1-180B	2.6	10.9	K1-200B	1.5	6.7	Y1-201A	2.9	10.7
K1-182A	3.2	5.2	K1-201A	1.3	11.3	Y1-201B	3.4	6.4
K1-182B	2.2	10.8	K1-201B	4.7	4.6	Y1-201C	3.8	4.7
K1-182C	1.9	8.5	K1-201C	2.4	5.1	Y1-201D	3.2	13.8
K1-182D	3.3	11.9	K1-201D	1.9	5.7	Y1-201E	2.0	11.1
K1-182E	3.2	10.5	K1-201E	5.5	8.4	Y1-201F	2.2	9.3
K1-182F	2.5	6.0	K3-183A	2.0	6.3	Y3-201A	1.5	7.7
K1-183A	1.1	12.1	K3-183B	3.8	5.1	Y3-201B	2.1	9.7
K1-183B	6.7	5.4	K3-183C	3.1	6.2	Y3-201C	2.4	13.3
K1-183C	1.1	4.7	K3-199A	2.4	8.1			
K1-183D	3.0	6.3	K3-199B	2.7	5.1	Mean	3.0	8.1
K1-186A	2.1	4.9	K3-201A	1.4	4.7			
K1-186B	2.9	6.7	K3-201B	1.6	7.1			
K1-186C	2.3	9.8	K3-201C	3.1	6.8			

For sample identification refer to Table No. 4.

Colony size. In the experiments in which milk samples were plated on the 7 special agars and the 73 stockpile agars, colony size seemed to be dependent upon nutritive qualities associated with the agars. It seemed desirable, therefore, to explore this possibility further, employing a pure culture so that the

colonial potentialities would be more uniform than in the case of milk. To this end two sets of nutrient broth plates were poured in triplicate with each of the 73 government stockpile agars. The first set was inoculated so that the count after 48 hours of incubation would be between 50 and 100 colonies; the second set between 200 and 300 colonies. From each plate, 10 random colonies were measured to obtain an estimate of the average colony size.

In Table 10 are the data obtained with a culture of Escherichia coli, strain 229. It is apparent from the data that the size of the colony is dependent upon the degree of crowding on the plate. In almost every instance the larger colonies appeared on the plates seeded with the smaller amount of inoculum. This is apparently at variance with the experiment in which the 73 agars were inoculated with milk, in which high counts, indicating crowding, were associated with a higher percentage of colonies over one mm. in diameter. In possible explanation, the hypothesis is advanced that on the high count plates many colonies may be more than one mm. in diameter but few reach the larger size attained by a relatively few large colonies on less crowded plates having a presumably lower nutritive level.

In order to determine which factor, nutrition or crowding was more important in restricting colony size, a series of experiments was started under similar conditions to those used with the seven test agars. The medium employed was Nutrient Agar with supplementary vitamins as used in the milk plate experiments. Again strain 229, E. coli was the test organism.

The results obtained are presented in Table 11. From these data it appears that riboflavin and nicotinic acid accentuate the effect of crowding on colony size. The mixture of vitamins, nicotinic acid and to a lesser extent calcium pantothenate apparently increased colony size on both high and low count plates while thiamine had a definite depressant effect on colony size, in this series.

Hemolysis. When tested as sterile media, only 2 out of the 73 government stockpile agars, and none of the 7 test agars or the Gracilaria extracts proved to be hemolytic. These 2 agars did not produce prompt hemolysis, but sterile blood agar plates prepared with whole rabbit blood were completely hemolyzed after incubation at 37°C. for 12 hours.

When cultures which normally produce alpha or beta hemolysis were tested on all but the two autohemolytic agars, no alteration in hemolytic type was observed. Serial transfer on the test agars and Gracilaria extract for 10 serial passages failed to alter the type of hemolysis produced.

Colonial and cultural characteristics. The growth characteristics of representative strains of bacteria belonging to the Escherichia, Aerobacter, Pseudomonas, Staphylococcus, Streptococcus and Neisseria genera were not altered by the various agars and Gracilaria. Of 69 such strains, 3 failed to grow on Gracilaria media; 1 of these was an alpha hemolytic streptococcus and the other 2 were strains of Pseudomonas. Of 32 strains of gram negative bacteria, 23 showed marked S-R variations on the Gracilaria medium, whereas only 4 of the 32 displayed a similar dissociation on the test agars.

TABLE 11. Effect of crowding and vitamin supplements on colony size of *Escherichia coli* grown on nutrient agar

Agar sample	Count	Average diameter in millimeters of ten random colonies					
		None	Thiamine 1/	Riboflavin	Calcium pantothenate	Nicotinic acid	Mixture
A	high	3.6	1.1	2.8	11.7	10.3	10.8
	low	6.0	3.3	5.5	10.3	22.1	10.8
B	high	4.2	1.8	2.3	9.8	10.2	8.6
	low	5.5	2.7	8.8	10.2	13.9	-
C	high	4.3	-	3.5	4.1	8.7	20.0
	low	6.0	-	7.6	5.0	15.6	8.0
D	high	4.2	2.8	3.7	5.9	8.3	13.1
	low	5.2	3.1	5.7	6.4	14.5	14.0
E	high	4.6	2.7	3.3	6.3	4.8	14.0
	low	4.8	3.0	7.0	6.2	-	18.9
F	high	3.8	3.4	3.3	6.0	5.9	10.1
	low	4.3	3.5	7.1	8.4	8.8	8.6
G	high	3.2	3.3	3.7	7.4	-	9.9
	low	4.6	2.8	6.8	7.9	-	9.2

1/ Concentrations same as reported in Table No. 8.

Pigment production by members of the genus *Pseudomonas* was greatly enhanced on nutrient medium prepared with *Gracilaria* extracts. Whereas only two out of the six strains tested showed the characteristic pigment of *Pseudomonas aeruginosa* on the test agars, all six produced a brilliant green pigment when grown on *Gracilaria* gels. A *Gracilaria* medium also enhanced photogenesis in the 10 strains of photogenic bacteria tested, the phosphorescent glow appearing sooner and lasting longer than with agar media.

When eosin methylene blue medium was streaked with cultures of characteristic *Escherichia* and *Aerobacter* species, the majority of the colonies appearing on the *Gracilaria* medium were atypical (15 out of 24 atypical after 24 hours at 37°C.; 11 atypical after 48 hours). On the same medium prepared with the test agars, all strains yielded colony types characteristic of the species.

Twelve strains of enterotoxigenic staphylococci were tested on Stone's medium for gelatin digestion. Five of these cultures yielded positive Stone's reactions, the average width of the zone being 3.2 mm. when the medium was prepared with the test agars. When prepared with *Gracilaria* extract, only two of the positive strains showed the zonation phenomenon, and the average zone width for these was but one mm.

All of the 69 strains of the genera listed above were transferred weekly for 10 serial passages on suitable media prepared with the test agars and *Gracilaria* extract. At the end of this time, the key cultural reactions were determined and compared with the initial characteristics determined at the beginning of the experiment. The serial passage failed to alter the cultural reactions of any of the organisms.

Tests were made with several strains of cellulose-digesting bacteria and fungi to determine whether Gracilaria might be attacked. The tests were all negative except that four out of six strains of Pseudomonas liquifying cultures showed no capacity for cellulose digestion.

Supplementary tests. A simple test was used for judging roughly the firmness of agar gels. Three persons compared texture of gels by the finger-rubbing technic. The results of this test were not consistent because of the inability to distinguish gradations of firmness (see Table 12). There was fairly good agreement in most cases when obviously firm or very weak agar gels were tested, but accord was lacking in the case of intermediate gels.

TABLE 12. Firmness of various agars scored by investigators X, Y, and Z by rubbing finger across surface of plate

Random agar samples	Investigators scores: Agar in distilled water			Nutrient agar		
	X	Y	Z	X	Y	Z
1	1	1	4	2	2	4
2	4	3	3	4	4	4
3	-	-	-	4	2	3
4	4	4	4	4	4	4
5	1	1	1	3	4	3
6	1	1	1	1	1	1
7	4	4	4	4	4	4
8	2	3	3	5	5	4
9	3	2	2	4	3	5
10	1	3	3	4	2	4

Scores:

--- not tested
1 very soft
2 very soft
3 is medium firm or intermediate
4 is firm
5 very firm.

Discussion

These studies indicate that within limits, the characteristics of agar derived from Gelidium and other closely related seaweeds are compatible with usual bacteriological practice. Limited work with extracts from Gracilaria confervoides (North Carolina) and with gums extracted from other sources of possible agar substitutes such as Irish moss, is sufficient to show that these gums are of a nature which makes them generally unsuitable as a substitute for agar in standard bacteriological culture media (Anzulovic, 1942; Stoloff, 1943b and Lee and Stoloff, 1946).

Variations in plate counts on media prepared with different agars seem to be due to the agar per se when the media contains less than critical amounts of certain nutrients. This seems to be the case with the current Standard Methods medium for plate counts on milk. There were indications that by increasing the nutrient quality with a liver extract or certain vitamins, variability in the counts deriving from differences in the agar can be reduced. Additional work with a larger group of agars and with a greater dilution of inoculum is necessary to show the extent of this equalization.

The use of liver extract and vitamins was an outgrowth of the work of Tittsler and Rugosa (1944). These investigators had observed that it was frequently necessary to carefully wash agar before using it in media for vitamin studies. This treatment was necessary in order to eliminate traces of vitamins present in certain samples of agar. Their studies of the effect of vitamins on various bacteria led these workers to suggest the use of liver extract and vitamins in these agar investigations. The fact that these nutritional supplements stimulated growth and increased colony size indicates that their inclusion in many plating media may be desirable.

The question has been raised as to whether the selection of 10 random colonies from a plate containing over 100 colonies represents an adequate sampling. Actually the number of colonies selected represents the maximum which could be accurately measured at the time. In all, over 5,000 individual colonies were measured and it was felt that the use of 10 colonies represented the situation as accurately as necessary for this work.

The properties of extracts from Gracilaria confervoides (North Carolina) are such that its use is impractical in bacteriology. The extracts gel at an excessively high temperature, about 60°C. This makes the material difficult to manipulate in addition to killing a number of bacteria which survive in ordinary agar media. The high gelling temperature results in an excess of condensation moisture, and this, in addition to the abundant water of syneresis, makes it impossible to use Gracilaria extract in media for either poured or streaked plates.

The characteristics of pure cultures grown on Gracilaria extract media did not change over a series of transfers. However, certain cultures failed to grow on such media. Also, there was a marked increase in S-R variations on Gracilaria media as compared to agar. For photogenesis and pigment production, Gracilaria is apparently superior to agar. The liquefaction of Gracilaria gels

by some strains of Pseudomonas might serve as a taxonomic character for that particular genus.

The finger test for firmness is of doubtful value. There was a general agreement when a particular gel was either very firm or very weak but there was no agreement with a gel between these extremes.

Conclusions

The special group of bacteriolcgical agars, as well as the stockpile agars, met the general requirements for a satisfactory bacteriological agar.

In general, the agars did not cause any cultural or morphological changes in a large number of strains of several genera of bacteria grown on them. In addition, all agars met the physical requirements for a bacteriological agar.

Both groups of agars contained unknown substances which affected, to a varying extent, the plate count and the average size of the colonies growing on the test medium. The colony size was also affected by the plate count, or degree of crowding, so that the relative importance of the two factors was difficult to estimate.

Addition of several vitamins, alone and in mixture, to the media did not lead to definite identification of the growth-stimulating substance present in some agars.

On the basis of comparative results obtained with one sample of extract from Gracilaria confervoides (North Carolina) this material is not suitable for use with either poured or streaked plates. It did have certain special properties, being a desirable media for pigment production by certain of the genus Pseudomonas, also enhancing the photogenic effect of 10 strains of photogenic bacteria tested.

No special restrictions or tolerances for bacteriological agar of an exclusively bacteriological nature are necessary, as chemical and physical properties can restrict the gums used to certain species of Gelidium. It would appear desirable, however, to study further the nutritive factors responsible for differences in plate count for different agars, with a view to modification of media formula so as to obtain uniform reponse, or optimum growth, not influenced by differences in the agar base used.

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